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Dissertação

Mestrado em Bioquímica

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2013

Trabalho dedicado a todas as pessoas que acreditam em mim e fazem com que eu tenha vontade de fazer sempre mais e melhor, a cada dia que passa.

RESUMO

A adenosina é um metabolito existente em todas as células e, tem um papel importante na função neuronal. Actualmente crê-se que este metabolito tem não só um papel como neuromodulador, mas também como regulador homeostático. O conceito de neuromodulador está relacionado com a capacidade de uma substância endógena, libertada na fenda sináptica, influenciar a libertação (modulação pré-sináptica) ou a acção (modulação pós-sináptica) do neurotransmissor. A adenosina é considerada um neuromodulador, na medida em que é responsável pela modulação da libertação de neurotransmissores, resposta pós-sináptica, bem como a acção de outros sistemas de receptores. Este metabolito é também um regulador homeostático pois, está envolvido na síntese de ácidos-base, metabolismo de aminoácidos e modulação do status metabólico da célula. Em condições normais, a adenosina é sintetizada tanto a nível intra como extracellular.

A família de receptores ligados a proteínas G, é a família de receptores aos quais a adenosina se liga, sendo conhecidos quatro tipos de receptores de adenosina: A_1, A_{2A}, A_{2B} e A_3 mas, neste trabalho irão ser apenas estudados os A_{2A}.

Os receptores A_{2A}, ao contrário dos A_1, apresentam uma restrita distribuição no cérebro, sendo característicos de regiões ricas em dopamina, sendo expressos nos neurónios GABAérgicos do corpo estriado. Através de estudos de imunohistoquímica, baixos níveis destes receptores foram detectados no hipocampo. Mesmo apresentando uma baixa expressão e densidade, estes são importantes nesta área do cérebro, desempenhando um papel importante na modulação da transmissão sináptica. É ainda importante salientar que, nesta área do cérebro os receptores encontram-se essencialmente nos terminais nervosos. Os receptores de adenosina A_{2A} têm ainda a particularidade de produzirem efeitos excitatórios, levando a uma activação da adenilato ciclase, a um aumento do AMP cíclico (cAMP) e a uma activação da proteína cinase A (PKA), culminando na promoção da libertação de glutamato.

Existem evidências de que um aumento dos níveis de expressão e função destes receptores A_{2A}, a nível do córtex e hipocampo, está associado a disfunção da função sináptica. Esta disfunção é também acompanhada por diminuição do desempenho em testes comportamentais em tarefas dependentes de hipocampo. No entanto, não se sabe se esses défices observados são gerados graças à sobreexpressão pré- ou pós-sináptica dos receptores A_{2A}.

Assim, o objectivo deste trabalho foi o de estudar em que região/área do cérebro ocorre a sobreexpressão dos receptores de adenosina A_{2A}. Levaram-se a cabo estudos de imunohistoquímca em secções de cérebro, e avaliou-se a localização subcellular dos receptores de adenosina A_{2A} usando técnicas de fraccionamento cellular e Western Blot. Para tal, o modelo utilizado foi o de ratos Sprague-Dawley transgénicos (8-16 semanas de idade), que sobreexpressam o receptor A_{2A} humano nos neurónios, sob o controlo do promotor CAMKII (CAMKII-hA_{2A}), e respectivos controlos *wild type*. Estes ratos apresentam défices de memória e de aprendizagem sendo apropriados para o objectivo do estudo.

Nos estudos de imunohistoquímica foram usadas secções coronais de cérebro, seccionadas no crióstato (20 µm) após um processo de perfusão, fixação com paraformaldeído e por fim gelatinização. Depois de cortadas, foram marcadas com um anticorpo específico para neurónios (Microtuble Associated Protein - MAP-2), núcleos (Hoechst) e para o receptor de interesse A_{2A}. Apesar da fraca marcação dos receptores A_{2A}, nota-se uma preferência de expressão dos receptores em torno dos corpos cellulares e ao longo dos neurónios (marcados com MAP-2).

Para os estudos da localização subcellular, recorreu-se a sinaptosomas plaqueados de hipocampo dos mesmos animais. Estas estruturas funcionam como um modelo de neurónio pré-sináptico, contendo o terminal pré-sináptico completo, a membrana pós-sináptica e a densidade pós-sináptica. Após a preparação dos sinaptossomas, procedeu-se a um ensaio de imunocitoquímica, onde os terminais pré-sinápticos foram marcados com sinaptofisina, uma proteína localizada nas vesículas sinápticas. A fracção pós-sináptica foi marcada com Post-Synaptic Density Protein (PSD-95), uma proteína pós sináptica, característica dos terminais glutamatérgicos. O

número de elementos marcados com sinaptofisina foi considerado como o número total de sinaptossomas. No hipocampo, a preparação de animais transgénicos possui 53.8±7.8% (n=4) de receptores A_{2A} em relação aos *wild type* - 10.5±6.1% (n=4). Detectou-se uma percentagem de receptores A_{2A} co-localizados com sinaptofisina de 39.3±6.7% (n=4) nos ratos transgénicos, e de 10.5±4.0% (n=4) dos ratos *wild type*. Os ratos transgénicos têm 10.8±2.3% (n=4) de receptores de adenosina A_{2A} co-localizados com PSD-95, comparando com 3.0±0.4% (n=4) nos *wild type*. No corpo estriado, observou-se também um enriquecimento de receptores de adenosina A_{2A} de 19.5±8.5% (n=4) nos *wild type* para 31.7±7.9% (n=4) nos transgénicos. Destes, 16.7±1.8% (n=4) co-localizam com sinaptofisina nos transgénicos e 6.5±1.5% (n=4) nos *wild type*. Por sua vez, 7.3±4.3% (n=4) dos sinaptossomas co-localizam com PSD-95 nos transgénicos e 6.0±1% (n=4) nos *wild type*.

Para completar os estudos de localização subcellular, foi realizado um protocolo de fracionamento sináptico onde, através de sucessivas centrifugações foi possível fazer um isolamento da fracção pré e pós-sináptica, em estriado e hipocampo de ratos *wild type* e transgénicos. Analisou-se a presença dos receptores de adenosina A_{2A} em ambas as fracções, por *Western Blotting*. No hipocampo observou-se, tal como nos ensaios de imunocitoquímica, que os receptores de adenosina A_{2A} são essencialmente pré-sinápticos (3.1±0.9%, n=5) comparando com a presença de receptores a nível pós-sinápticos (2.6±1.6%, n=5). No estriado, observou-se para os receptores de adenosina A_{2A} uma preferência pela localização pós-sináptica (9.6±0.6%, n=2) comparando com a pré-sináptica (2.5±0.6%, n=3).

Em conclusão, os resultados deste trabalho mostram que no hipocampo a sobreexpressão dos receptores de adenosina A_{2A} é essencialmente pré-sináptica. Esta localização está de acordo com os estudos funcionais no envelhecimento e em situações de stress crónico onde o aumento pré-sináptico dos receptores de adenosina A_{2A} é apontado como o desencadeador de uma alteração na modulação pela adenosina no hipocampo .

ABSTRACT

A_{2A} receptors are constitutively activated G-protein coupled-receptors (GPCRs), preferentially expressed by the striatopallidal medium spiny striatal neurons. They exhibit however a very distinct pattern of expression in the hippocampus and cortex where their expression is very low in physiological conditions and mainly in the nerve terminal. Our team and others have found compelling evidence of cortical and hippocampal upsurge of A_{2A} receptors expression/function associated to cognitive deficits. This is accompanied by clear behavioral deficits in hippocampal-dependent tasks, such as spatial memory in rats.

However, the mechanism by which A_{2A} receptors dysregulation drives synaptic, cognitive and Alzheimer´s Disease-related pathological hallmarks is unknown; and whether these deficits, are generated by A_{2A} receptors located pre- or post-synaptically remains to be uncovered.

We now used transgenic rats overexpressing human A_{2A} receptors in forebrain, under CAMKII promoter (CAMKII-hA_{2A}), which display learning and memory deficits. In order to verify if these deficits are related to pre- or post-synaptic A_{2A} receptors overexpression, we performed a sequential cellular fractionation protocol, using hippocampi derived from wild type (wt) and transgenic (tg) male rats. We were able to efficiently separate the pre- and post- synaptic fractions, as assessed by obtaining exclusive positive Synaptosomal-Associated Protein 25 (SNAP-25) or PSD-95, respectively. The total level of A_{2A} receptors was increased by 3.3±0.7 fold in tg versus wt rats (n=4). In both wt and tg, A_{2A} receptors were found preferentially located in SNAP-25 positive fractions comparing to PSD-95 positive fraction (3.1±0.9 versus 2.7±1.6%, n=5). As a control, we assessed striatum and as expected we observed a preferential postsynaptic localization both in wt and tg animals. We also confirmed co-localization of A_{2A} receptors with synaptophysin in the hippocampus, by using immmunocytochemistry in plated synaptosomes derived from the same animals (10.5±4.0% versus 39.3±6.7%, n=4). This shows that A_{2A} receptors overexpression in

the hippocampus occurs preferentially in the nerve terminal, rather than in the postsynaptic density.

The pre-synaptic location of A_{2A} receptors in these animals is in agreement with electrophysiology and behavior results, and follows the age progressive physiological overexpression in the hippocampus.

Keywords: adenosine, A_{2A} receptors, hippocampus, synaptosomes, pre-synaptic, post-synaptic

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A1R	Adenosine A_1 Receptor
$A_{2A}R$	Adenosine A_{2A} Receptor
ADA	Adenosine Deaminase
AK	Adenosine Kinase
AMP	Adenosine Monophosphate
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AR	Adenosine Receptors
cAMP	Cyclic Adenosine Monophosphate
CGS	3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid
GABA	γ-Aminobutyric acid
GFAP	Glial Fibrillary Acidic Protein
GPCRs	G Protein Coupled Receptors
LTP	Long Term Potentiation
MAP-2	Microtubule Associated Protein-2
PKA	Protein Kinase A
PLC	Phohpholipase C
PSD-95	Post Synaptic Density Protein 95
SCH	5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine
SNAP-25	Synaptosomal-Associated Protein 25
ZM	4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

Introduction

The nervous system is the most complex biological system in the human body and is unique in the vast complexity of thought processes and control actions it can perform. Each minute it receives millions of bits of information from different sensory nerves and sensory organs and then integrates all these to determine responses to be made by the body. It is composed by brain, spinal cord and peripheral nerves.

There are about 100 billion nerve cells in the brain, and on average each of these cells communicates directly with 1000 others.

The mechanism of conversion of the pre-synaptic spike, or a sequence of spikes, into a graded variation of the post-synaptic membrane potential that is another form of codification of the same information (synaptic coding of the information) is called synaptic transmission [1].

Some debates started because of the origin of this communication across synapses between nerve cells. Ones believed that it can be electrical, where the nerve impulse or action potential is propagated along the axon to the nerve ending, changing the electric field across plasma membrane of the post-synaptic cell and thereby produce a physiological response; others believe it is chemical, where when the action potential came down the axon to nerve terminal cause the fusion of neurotransmitter-containing vesicles with the pre-synaptic plasma membrane, releasing the neurotransmitter which then diffuse across the synaptic cleft and through activation of a receptor, produce a physiological response [2].

This information flow and processing is regulated by a class of substances, the neuromodulators, where adenosine belongs [3].

Adenosine in the nervous system

Adenosine is a purine nucleoside [4] and the interest on its study started 70 years ago [5]. Now, adenosine is considered as a constitutive metabolite of all cells [6] and it can act not only as a neuromodulator but also as an homeostatic modulator [7, 8]. The concept of neuromodulator is related to the capacity of an endogenous substance, released at the synaptic cleft, influence the release (pre-synaptic modulation) or the action (post-synaptic modulation) of the neurotransmitter [3].

Adenosine, in normal conditions, is formed both intracellularly and extracellularly [9]. Taking into account the intracellular formation, this one is via two distinct metabolic pathways: hydrolysis of AMP by an endo 5´-nucleotidase [10] and catabolism of S-adenosylhomocystein by hydrolysis of S-adenosil homocystein (figure 1.1) [11]. Judging from the kinetic constants of some of the major enzymes involved, the intracellular concentration of adenosine at equilibrium is probably around 100nM [12]. This concentration value is controlled by the rapid phosphorylation of adenosine by adenosine kinase and also by its convertion to inosine by adenosine deaminase and consequent conversion to hypoxanthine by nucleoside phosphorylase [13, 14].

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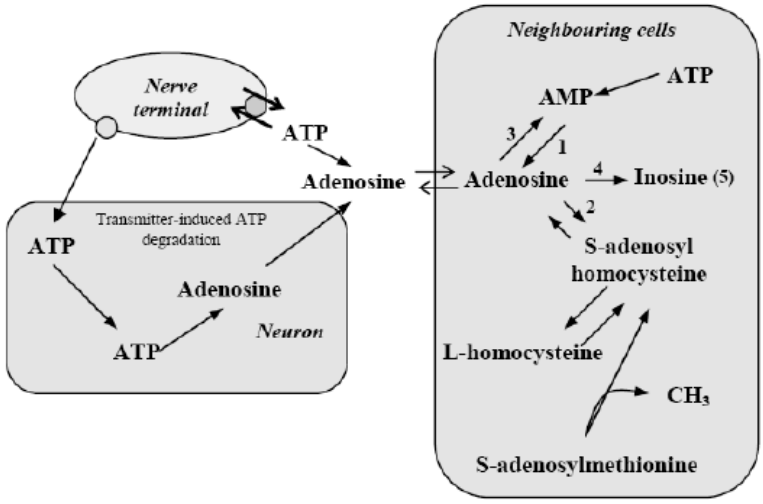


Figure 1.1 - Mechanisms for intracellular metabolism of adenosine. reactions represented are catalyzed by the enzymes: 1.5'-endonucleotidase, 2. S-adenosil homocystein hydrolase, 3. Adenosine kinase, 4.adenosine deaminase, 5.nucleoside phosphorylase **[15]**

The main pathway of adenosine metabolism, that is more pronounced in neurons than in glial cells [16], is its incorporation into nucleotides as predicted by the difference between K_m of Adenosine Deaminase (ADA) (14-45 µM) and the k_m of Adenosine Kinase (AK) (2µM) [10]. In stressful situations such as hypoxia and hypoglicaemia, intracellular concentration of adenosine increases up to 100-fold [17].

The concentration of extracellular endogenous adenosine is around 140-200nM [18] but, in stressful situation as mentioned before these values rise 10 and 3 times more, respectively. This extracellular adenosine is thought to be generated either as desphosphorylation of adenosine nucleotides by ecto-nucleotidases or

released adenosine and, it can be transported mainly via specific bi-directional transporters through facilitated diffusion [6, 19]. This adenosine is removed from extracellular space by two processes: deamination or transport to the interior of cells and subsequent metabolisation [20].

Adenosine receptors

In the early 1970s, the development of specific radiolabeled ligands and the establishment of suitable methods, opened the era of molecular studies of the transmembrane receptors, allowing an approach to isolation and characterization of the receptors [21].

The family of receptors coupled to heterotrimeric G proteins, also known as GPCRs or seven transmembrane domain receptors, that adenosine binds to, are named adenosine receptors (ARs) [22]. There are four types of AR designated A_1, A_{2A}, A_{2B} and A_3 and these all four types are N-linked glycoproteins [23] and, all have been cloned and characterized from several mammalian species including humans.

Only adenosine A_1 and A_{2A} receptors are both abundantly expressed in the central nervous system and have high affinity for adenosine to be activated by physiological levels of this nucleoside [24].

Adenosine A_1 receptor (A_1R), that is the adenosine receptor with the highest density in the hippocampus, cerebral cortex, cerebellum and some thalamic nuclei [25] is the most abundant and homogeneously distributed AR in the brain. At cellular level they are located pre-, post- and non-synaptically .It is an inhibitory receptor which is functionally coupled to members of the pertussis toxin-sensitive family of G-proteins ($G_{\alpha i/o}$) and whose activation leads to an inhibition of the activity of the enzyme adenylate cyclase, to a regulation of Ca^+ channels, K^+ channels and Phospholipase C (PLC) [26, 27].

The activation of these receptors reduces long-term changes in synaptic efficiency, such as Long Term Potentiation (LTP) and, the release of glutamate and aspartate from nerve terminals in the CA1 region of the hippocampus is highly sensitive to inhibition by adenosine [28].

Adenosine A_{2A} receptors

Unlike A_1R, adenosine A_{2A} receptors ($A_{2A}R$) show a more restricted distribution, being characteristic of dopamine enriched areas and highly expressed in the striato-pallidal GABAergic neurons. Studies with autoradiography using the agonist ligand ((2-[p-(2-carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamido adenosine) (CGS) allowed high staining levels in striatum, both in dorsal and ventral (nucleus accumbens). Within this brain region these receptors are particularly abundant in plasma membrane of dendrites and denditric spines and less in axon and axon terminals and glial cells [29] and in olfactory tubercule and globus pallidus. From Northern Blot and *in situ* hybridization studies it was further evident that striatal $A_{2A}R$ was mostly exclusively expressed in the medium spiny neurons, co localizing with enkephalin and dopamine D_2 receptors containing cells [30]. By means of more sensible techniques, like immunohistochemistry or radioligand binding, lower levels of $A_{2A}R$ were also detected in other parts of the brain as amygdala, hippocampus, hypothalamus, thalamus and cerebellum [31]. But, in spite of the low expression and density of $A_{2A}R$ in the hippocampus, they play an important role in the modulation of synaptic transmission [32].

$A_{2A}R$ are pleiotropic, mostly coupling to G_s proteins [33]. This coupling result in adenylyl cyclase activation, increase in cellular cAMP levels and PKA activation [34]. Due to this, $A_{2A}R$ are called excitatory, unlike A_1 which are inhibitory. Activation of the $A_{2A}R$-G_s signal transduction pathway appears to be involved in adenosine-induced vasodilatation, inhibition of platelet aggregation, modulation of neutrophil function [35] and inhibit voltage-dependent Na^+ channels through a cyclic AMP-dependent mechanism.

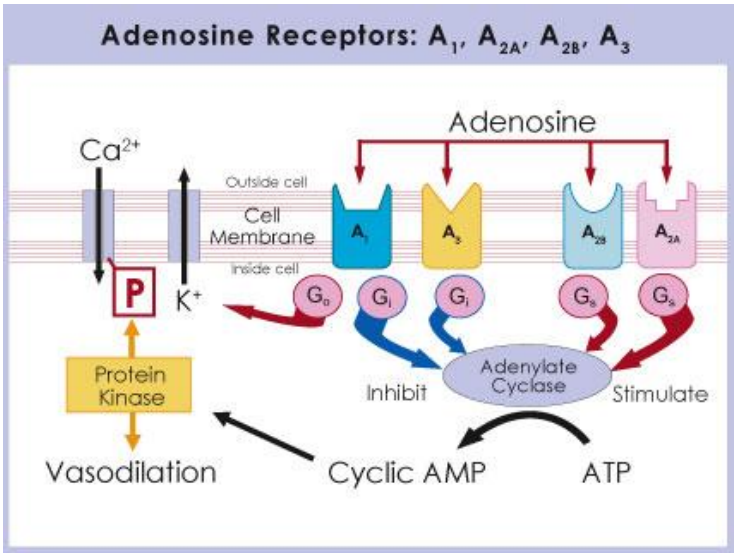


Figure 1.2. – Adenosine receptor signaling. Adenosine mediates its action via four G-protein coupled receptors, A_1, A_{2A}, A_{2B}, and A_3 that are coupled primarily to the activation and inhibition of cAMP. (Adapted from Ham J. , 2012)

$A_{2A}R$ can be found both pre- and post- synaptically: pre-synaptically on the corticostriatal glutamatergic (excitatory) projections and post-synaptically on the GABAergic (inhibitory) striatopallidal neurons [29]. Pre-synaptic $A_{2A}R$ modulates A_1 receptor inhibitory actions, resulting in facilitatory effect on synaptic transmission [36], modulates the release or uptake of neurotransmitters, namely glutamate [37], GABA [38] and acetylcholine [39]. Post-synaptically, $A_{2A}R$ are implicated in the modulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) currents [40]. There are also evidences that post-synaptically $A_{2A}R$ can mediate neuroprotection by potentiating Brain-derived Neurotrophic factor (BDNF) survival signaling pathways [41]. In terms of astrocytes $A_{2A}R$ are involved in the modulation of glutamate release [42] and GABA uptake.

To study the effects of the activation of these receptors is useful to know the pharmacology behind activation and inhibition processes. NECA was long considered to be a selective adenosine A_2 receptor agonist but now, based on evidence that 2-substitution of NECA increased selectivity, 3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid (CGS 21680) was developed as an $A_{2A}R$ selective agonist [43]. However, in humans

it is less potent and less selective than in rats. In terms of useful $A_{2A}R$ antagonists, the most selective so far is 5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine (SCH 58261) but, 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385) considered as a novel non-xanthine adenosine receptor antagonist [44].

In the early 1980s some researchers proposed a direct molecular interaction between receptors for neurotransmitters and neuromodulators [45]. Ten years later, was shown that dopamine receptors occurred as dimmers when expressed in certain type of cells. The most well known heterodimeric interaction involving $A_{2A}R$ is with dopamine $D_2R.A_{2A}/D_2$ receptor heteromers have been proposed to be a distinct target for therapy in Parkinson's Disease, for example [46]. Between these two receptors there is an antagonistic interaction at the membrane and functional levels. Therefore the blockade of $A_{2A}R$ mimics the action of D_2 agonists [47].

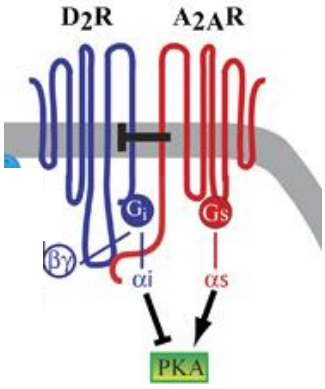


Figure 1.3– Schematic representation of $D_2/A_{2A}R$ interactions. (Adapted from Azdad K., 2009)

In addition, it is interesting to explore the negative crosstalk between A_1 and A_{2A} receptors. This interaction is expected in regions having cells co-expressing both receptors, but not as much in regions (like cerebral cortex) where the expression of the $A_{2A}R$ is low and hence no heterodimerization would be antecipated. Thinking that A_{2A} receptors increase cAMP levels and A_1R decreases, it is impossible to have the two actions simultaneously [48]. A possible heterodimerization has the ability of, by activation of $A_{2A}R$, to reduce the affinity of the A_1R agonists, providing a switch

mechanism by which low and high concentrations of adenosine inhibit and stimulate, respectively, glutamatergic neurotransmission [49].

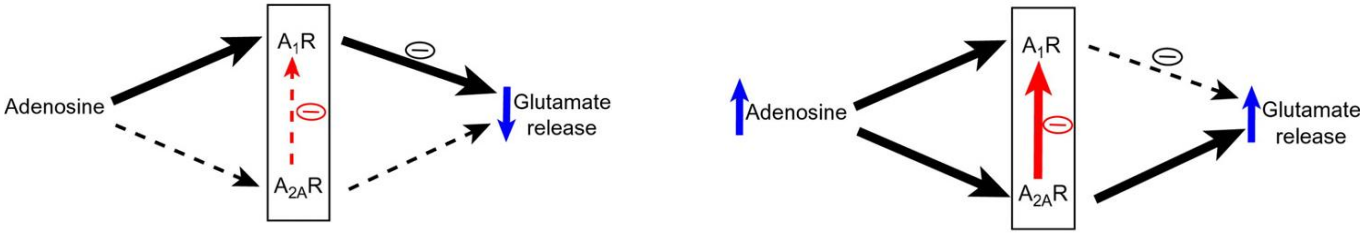


Figure 1.4 - Receptor-heteromer mediated dual regulation of glutamate release by adenosine. Adenosine A_1 receptors are coupled negatively to the adenylate cyclase whereas adenosine A_{2A} receptors are coupled positively to the cyclase. Coexpression of the two receptors in a given neuron gives rise to A_1/A_{2A} receptor heterodimers, which provide a different functional output in terms of regulation of glutamatergic neurotransmission in the striatum. At low concentrations adenosine depresses glutamate release in GABAergic striatal neurons. At high concentrations adenosine in the same neurons enhances glutamate release. This signaling via A_1 receptors at low adenosine concentrations and via A_{2A} receptors at high adenosine concentrations is only possible by the occurrence of presynaptic $A_1/_{A2A}$ receptor heteromers (Adapted from Franco R., 2008)

Hippocampus, ageing and memory

This structure of the medial temporal region of the brain is a structure with a distinct shape and cytoarchitecture, being a primary target for stress hormones involved in long-term memory formation [50], storage and retrieval of certain types of information. It is identified as a key mediating structure in studies examining the pathophysiology of disordered memory [51].

Hippocampal neuroanatomy is highly organized providing an unidirectional circuit, divided in 3 areas: dentate gyrus (DG), that processes metric spatial representation, CA3 that is responsible for spatial pattern association and short term memory and CA1 that mediates processes involved in temporal pattern association and completion as well as intermediate-term memory [52]. The information arises from the entorhinal cortex to the DG which axons (mossy fibers) innervate CA3 synapses. CA3 projects through Schaffer collaterals axons to CA1 and this back to the entorhinal cortex closing the unidirectional circuit of input integration [53].

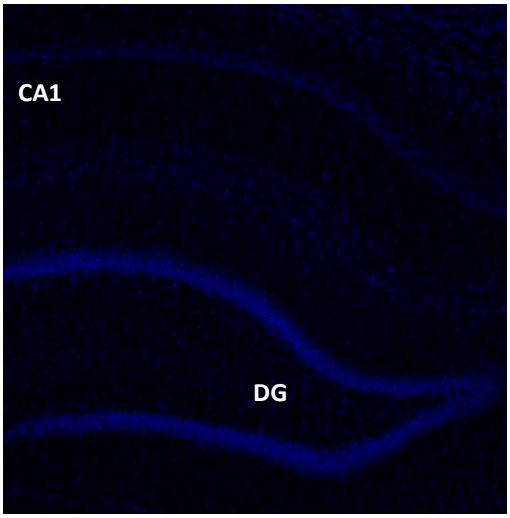


Figure 1.5. - Representative image, obtained by the author in confocal microscope (Zeiss 710) with 5x magnification, of two main areas of the hippocampus. Neurons nuclei were stained with Hoechst (blue)

Adenosine receptors in this brain area are modulators of synaptic transmission and neuronal excitability. Glutamatergic synaptic transmission in physiological conditions is controlled negatively by the dominant A_1R and positively to a lesser extent by $A_{2A}R$ [36].

Since this area is so important in many processes, if there is a damage in that, impairments in spatial and episodic memory will appear. Other important point is that upon aging, as well as upon chronic noxious brain conditions, adenosine modulation is modified with increased levels of adenosine, increased density and effects of $A_{2A}Rs$ and a decrease of A_1Rs [54]. There are studies that prove a compelling association between these modifications, namely the cortical and hippocampal upsurge of $A_{2A}R$ expression/function and consequent cognitive deficits [55].

AIM

Adenosine A_{2A} receptors are one of the main brain target of the homeostatic neuromodulator adenosine [56]. These receptors are GPCR, mainly expressed by the striato-pallidal medium spiny striatal neurons [57]. They are also present in other brain areas namely hippocampus, with a very distinct pattern of expression and where its expression is very low in physiological conditions and mainly in nerve terminals [58]

Our team and others have found compelling evidence of cortical and hippocampal upsurge of A_{2A} receptors expression/function associated to cognitive deficits [55]. Other studies found that an A_{2A} receptors overexpression in hippocampus cause behavioural deficits including spatial memory and LTP impairments [59]. However, it is not known whether these deficits are generated by adenosine A_{2A} receptors located pre- or post synaptically.

The aim of this work was to evaluate the subcellular localization of adenosine A_{2A} receptors and its profile of overexpression in transgenic rats overexpressing human A_{2A} receptors in forebrain, under CAMKII promoter (CAMKII-hA_{2A}), which display learning and memory deficits (Valadas et al, 2012). (VALADAS JS; COMIM CM; SHMIDT T; SEBASTIÃO AM; BADER M AND LOPES LV. Adenosine A_{2A} receptor overexpression in rat forebrain. FENS Forum, Barcelona, 2012)

We used the following approaches:

- Brain slices immunohistochemistry to ascertain adenosine A_{2A} receptor distribution profile;
- Plated synaptosomes immunocitochemistry to determine in which subcellular region the adenosine A_{2A} receptor overexpression is dominant;
- Western Blot in fractionated tissue, to determine subcellular localization.

Material and Methods

Animal Model

Transgenic rats overexpressing human A_{2A} receptors, under CAMKII promoter (tg CAMKII-hA_{2A}R) that were generated by microinjection of a DNA construct into the male pronucleous of Sprague-Dawley rat zygotes with established methods [60]. The rats used were males, with age between 8 and 16 weeks-old, and genotype confirmed for individual animals as follows: DNA was isolated from mouse ear by adding 50µL of Tail Digestion Buffer (TDB) and incubated overnight at 56°C then, in a dry bath heat inactivate for 15 minutes, spin sample for 2 minutes at max speed to get rid of debris and freeze sample. To perform Protein Chain Reaction (PCR), a Mix was prepared with ddH_2O, DreamTaq Buffer, dNTP, primers: Cam2a3, Cam2a5, Forward Actin (ActF) and Reverse Actin (ActR) and DreamTaq Polymerase. Then 24µL of this Mix was added in an eppendorf and then 2µL of DNA. Finally samples were put in Termocycler and, to analyze the experiment, an agarose gel was prepared and in the UV transluminator the gel can be seen. If we observe a band at 45 kDa it means that that mouse is tg (Figure 3.1)

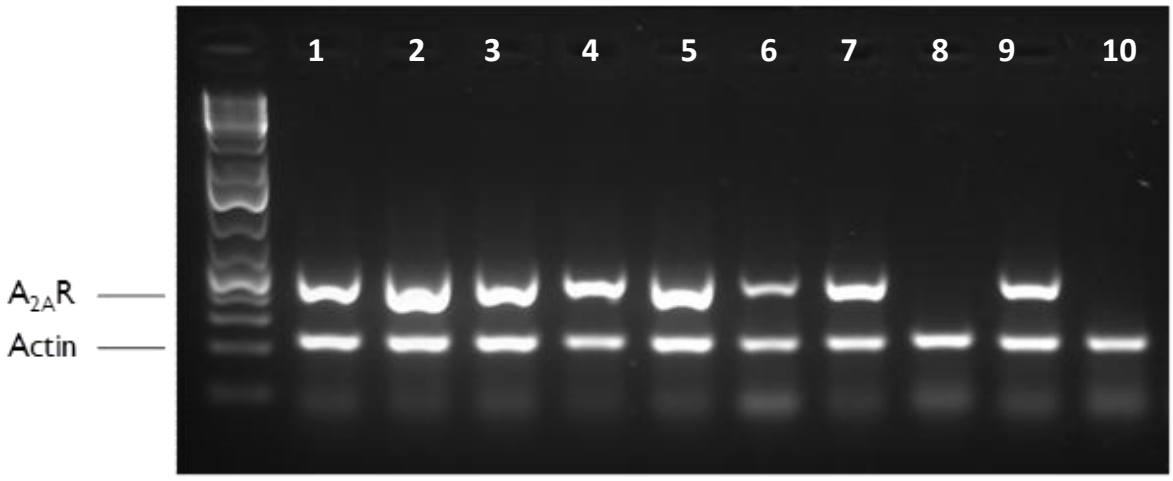


Figure 3.1 - Genotyping representative agarose gel for 10 tested animals, where the Actin bands (control) and the A_{2A}R positive signal can be identified..

Perfusion Protocol

The rat was anaesthetized with an intraperitonial injection (IP) of a mixture containing ketamine/xylazine and placed in a convex surface. A lateral incision just below the sternum was made to initialize the Xiphoid Process with forceps, to see the diaphragm. Using scissors diaphragm was cut, the thoracic cavity exposed and a longitudinal cut along sternum was made. A needle was inserted into left ventricle and hole was spined in right atrium. First the blood was washed with normal saline (NaCl solution: 9g of NaCl + 1L H_2O) for more or less 30 minutes and then tissues were fixed with 4% paraformaldyde in Phosphate Buffer Saline (PBS), composition in mM: NaCl 137; KCl 2.7; KH_2PO_4 1.5; Na2HPO_4 8) for 50 minutes.

After perfusion, rats were decapitated, brains were removed from cranium and stored in a falcon with a sucrose solution in PBS, first 15% and when the brain sink the bottom, the 15% solution was replaced by a 30% solution. According to [61]

Immunohistochemistry

To study the adenosine A_{2A} receptors localization in whole brain slices, mainly in hippocampal and striatal areas, an immunohistochemistry assay was performed, based on a previous implemented protocol [62]

To perform this procedure, brains that were in 30% sucrose solution must be embedded in a gelatin mix, to allow a posterior freezing. To prepare the gelatin mix, PBS (300mL) was warmed to 55°C and gelatin (7.5%, 22.5g) and sucrose (15%, 45g) powders were added slowly to PBS. Then, solution was equilibrated at 37°C, brains were placed on a falcon and incubated with gelatin/sucrose mix for 30 minutes to-1 hour at 37°C. To embed the brains, a box with high walls (minimum 5cm) was needed and the box was lined with aluminum foil. After that, a small layer of gelatin/sucrose mix was put and let it solidify at room temp. When it was firmed, the brains were set on top of this layer side by side and the rest of the mix was put on top, to cover the brains. Finally waited for the gelatin to solidify at room temperature, before placing it in fridge for at least 30 minutes.

After this, blocks needed to be frozen and for this, an amount of liquid nitrogen was put into a container. Isopentane into a plastic beaker was cooled in liquid nitrogen to -70°C to -80°C. When isopentane reached -70°C it was taken out of the liquid. When temperature started to rise slightly the blocks were immersed into the isopentane until the block is thoroughly frozen.

Frozen blocks were stored at -80°C for at least 24 hours after, they were cut using a Criostat Leica CM 3050S, in 20µm slices, to perform immunohistochemistry staining: First, slices were de-gelatinized 10 minutes with PBS and quenched with 0.1M glycine (for 250mL: 1.88g of glycine + 250mL PBS) for 20 minutes. To facilitate the antibody accessibility and prevent non-specific binding, at room temperature a permeabilization and blocking step was made, adding 400-500µL per slide of blocking solution: Fetal Bovine Serum (FBS) 10%, Bovine Serum Albumine (BSA) 1% in Tris Buffer Saline plus Tween 0.1% (TBST) for 1 hour. At this point, slices can be stained with primary antibodies (70µL per slide), over 48 hours at 4°C. Immunodetection was performed for Glial Fibrillary Acidic Protein (GFAP) with anti-GFAP from Chemicon (mouse, 1:100), for Microtubule-associated protein 2 (MAP-2) that stain mainly neurons with anti-MAP2 from Millipore (mouse, 1:500) and for Adenosine A_{2A} Receptors ($A_{2A}R$) with anti-$A_{2A}R$ from Millipore (goat, 1:200). After 48 hours of incubation, slices were washed three times for 15 minutes with TBST and slices were stained with secondary antibody overnight at 4°C then, washed again for 30 minutes with TBST and for nuclei identification, 10 minutes incubation with Hoeschst was performed. One last wash was performed with PBS for 10 minutes to remove Hoechst and the slides were mounted with Mowiol and let to dry overnight at room temperature, protected from light exposure. Fluorescent visualization was done with a fluorescent microscope (Inverted Widefield Fluorescence Microscope, Zeiss Axiovert 200M, Germany).

Fractionation Protocol

Animals were anesthetized under isofluorane atmosphere before decapitation. The brain was rapidly removed from the brain cavity and striata and hippocampi were dissected free in PBS solution. The tissues were homogenized in cold buffer (Sucrose 0,32M, HEPES 10mM pH 7.4) at Potter-Elvehjem. Next, a successive centrifugation protocol was made, started with a 1000g centrifugation for 10min, the pellet corresponds to nuclei and large debris, the supernatant was cleared twice and then centrifuged at 12000g for 20min, the obtained pellet corresponds to crude membrane fraction and at this pellet a solution of 4mM HEPES, 1mM EDTA pH 7.4 was added and centrifuged at 12000g for 20min, the pellet corresponds to synaptossomal membrane fraction and this was washed twice and then a 20mM HEPES, 100mM NaCl, 0,5% Triton-X100 ph=7.2 solution was added, the mixture was incubated at 4°C for 15min and centrifuged at 12000g for 20min, the obtained supernatant corresponds to non-post synaptic membrane fraction (non-PSD) and at the pellet a 20mM HEPES 0,15mM NaCl 1% deoxycholic acid 1% SDS pH=7.5 solution was added and the mixture incubated at 4°C for 1h and then centrifuged at 10000g for 15min. The obtained supernatant corresponds to the PSD.

Western Blot

After protein quantification using BioRad DC Protein assay Kit, based on Lowry (1951), the samples were diluted in water and sample buffer 5x (350 mM Tris pH 6.8, 30% glycerol, 10%SDS, 600 mM DTT and 0,012% Bromophenol blue). Prior to loading, samples were denaturated at 60°C for 20min. Protein samples and molecular weight marker were separated by SDS-PAGE (10% and 12%, according do protein molecular weight and a 5% stacking) in denaturating conditions and electro-transferred to PVDF membranes (Millipore). The percentage of resolving gels and protein loading amounts are summarized in Table 1. Membranes were blocked with 3% BSA for 1 hour and a half and incubated overnight at 4°C with primary antibody. After washing for 30 min with TBS-T (Tris Buffer Saline solution, 200nM Tris, 1.5 M NaCl with 0,1% Tween-20), the membranes were incubated with secondary antibody for 1 hour at room temperature. After 45 minutes of washing with TBS-T, chemoluminescent detection was performed with ECL and ECL-Plus western blot detection reagent (GE Healthcare) using X-Ray films (Fujifilm). Optical density

was determined with Image-J software and normalized to the respective pan-cadherin band density.

Synaptosomes preparation

In neurochemical research, it has been accepted to divide neuronal parts of the synapse into two: pre and post-synaptic [63]. Since the early 1960s, “pinched-off” pre-synaptic nerve terminals or “synaptosomes” have an extensively use as a model of pre-synaptic neuron. Rather than organelles, synaptosomes are artificial, membranous sacs that contain synaptic components and are generated by subcellular fractionation of homogenized or ground-up nerve tissue. Synaptosomes contain the complete pre-synaptic terminal, including mitochondria and synaptic vesicles, along with the post-synaptic membrane and the PSD [64].

In this work, to access subcellular localization of adenosine A_{2A} receptor, synaptosomes were prepared as before [65]. Rats were anesthetized as described above in fractionation protocol but, the brain areas were dissected in ice-cold Krebs/HEPES solution with the composition (mM): NaCl 124, Glucose 10, HEPES 25 KCl 3, $MgCl_2$ 1, $CaCl_2$ 2 pH 7.4. Then 2 hippocampi and striata were placed in a vial with 5 mL of 0.32 M sucrose solution containing 1 mM EDTA, 1 mg/mL BSA and 5 mM HEPES, pH 7.4, and homogenized in a Potter-Elvehjem homogenizer with a Teflon piston (3 up and down strokes). The volume of the suspension was completed to 10mL with sucrose solution and centrifuged at 3000g for 10 minutes at 4°C, the supernatant collected and centrifuged at 14000g for 12 minutes at 4°C. The pellet was ressuspended in 2mL of 45% v/v Percoll solution made in Krebs-Ringer solution (composition in mM: NaCl 140, KCl 5,HEPES 25, EDTA 1, Glucose 10, pH 7.4). After centrifugation at 14000g for 2 minutes the top layer was collected (synaptosomal fraction) and washed two times in KHR solution (composition in mM: NaCl 140, EDTA 100, HEPES 10, KCl 5, glucose 5) by ressuspension and subsequent centrifugation at 14000g for 2 minutes. Synaptosomes were used for immunocitochemistry assays.

Immunocitochemistry

Coverslips were covered with 100µL of poly-D lysine (0.166 M in borate buffer) for 1 hour at 37°C. Then, 100µL of synaptosomes were put on the coverslips, at 37°C for 3 hours, blocked with 4% paraformaldehyde for 15 minutes at room temperature, washed twice with PBS medium, incubated 10 minutes at room temperature with 0.2% Triton X-100 in PBS (120µL per coverslip) and then washed again, as in [66]. For the blocking step, synaptosomes were incubated with FBS 10% for 1 hour at room temperature and washed twice with PBS+0.05% Tween-20. Incubation with primary antibodies (diluted in FBS 10%) was performed overnight at 4°C and synaptosomes were washed three times with PBS+0.05% Tween-20 before incubation with secondary antibody from Life Technologies (donkey anti-mouse 568, donkey anti-rabbit 488 and donkey anti-goat 633, 1:250). Immunodetection was performed for pre-synaptic fraction (synaptophysin 1:250 from Sigma), post-synaptic fraction (PSD-95 1:250 from Cell Signalling) and adenosine A_{2A} receptors (1:200 from Upstate). One last wash was made three times with PBS, a mounting media (Dako form Patex media S.A.) was added in a slide and the coverslips were put there and dry overnight at room temperature, protected from light exposure. Fluorescence visualization was done with a fluorescence microscope (Inverted Widefield Fluorescence Microscope, Zeiss Axiovert 200M, Germany). The analysis of images was performed with ImageJ Software, using a macro, developed by Bioimaging Unit. The threshold applied to count stained elements was between 75-150.

Statistics

The values presented are mean ±SEM of n experiments. The n represents a pair of wt and tg rats. In statistical tests for three or more conditions, a one way ANOVA was used, followed by a Bonferroni's Multiple Comparison post hoc test. Values of $P<0.05$ were considered to be statistically significant. In other conditions, a unpaired Student´s T test was used. In this, the following code was used for A_{2A} immunoreactivity: *$P<0.01$. For synaptosomes experiments (ANOVA): *$P<0.0001$,#$P<0.05$, to compare each condition to the control.

Table 1 – Primary and secondary antibodies and related conditions used in Western Blot experiments for individual proteins. All primary antibodies were diluted in 3%BSA with 0.1%NaN_2 and secondary antibodies in 5% non-fat dry milk

Protein	Resolving Gel (%)	Primary antibody	Animal	Dilution	Secondary antibody	Dilution
$A_{2A}R$	10	Upstate	Mouse	1:2000	Santa Cruz	1:5000
PSD-95	10	Cell Sigmalling	Rabbit	1:10000	Biotechnology	1:10000
SNAP-25	10	Sigma	Rabbit	1:30000	(goat anti-mouse; goat	1:10000
Pan-cadherin	10	Abcam	Rabbit	1:30000	anti-rabbit	1:10000

Abbreviations: Adenosine A_{2A} receptors ($A_{2A}R$); Post-Synaptic Density (PSD-95); Synaptosomal-Associated Protein 25 (SNAP-25)

Results

Subcellular localization of Adenosine A_{2A} Receptors

To study the subcellular localization of adenosine A_{2A} receptors, pre-and post-synaptic fractions were separated with a fractionation protocol, based in a sucrose gradient (See Material and Methods).

To assess fractionation efficiency, Western Blot was performed and the total homogenate, pre- and post-synaptic fractions were tested with pre- and post-synaptic markers: SNAP-25, a pre-synaptic protein, and with PSD-95, a post-synaptic protein. In Figure 4.1 it is possible to observe a representative Western Blot that shows SNAP-25 enrichment in pre-synaptic fraction and PSD-95 enrichment in post-synaptic fraction. This control was made to every individual fractionation before subsequent use of fractions.

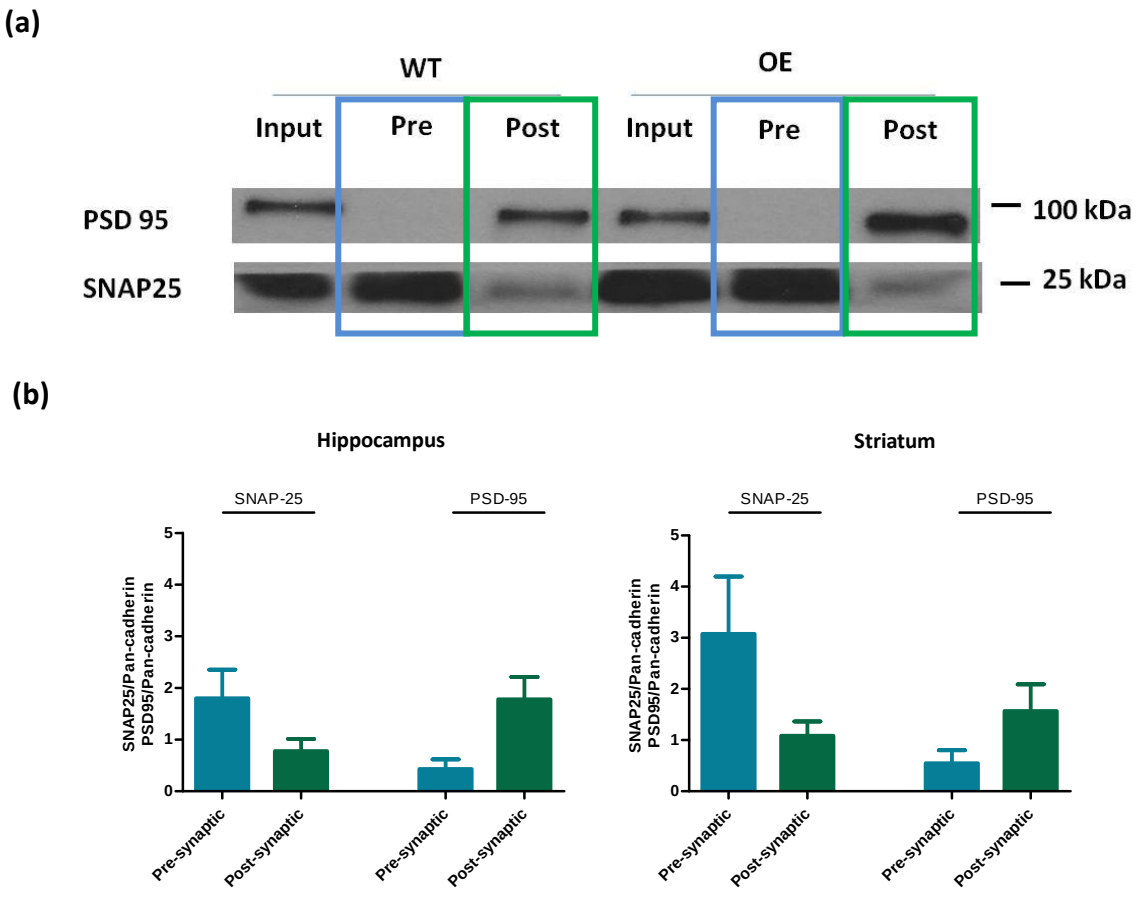
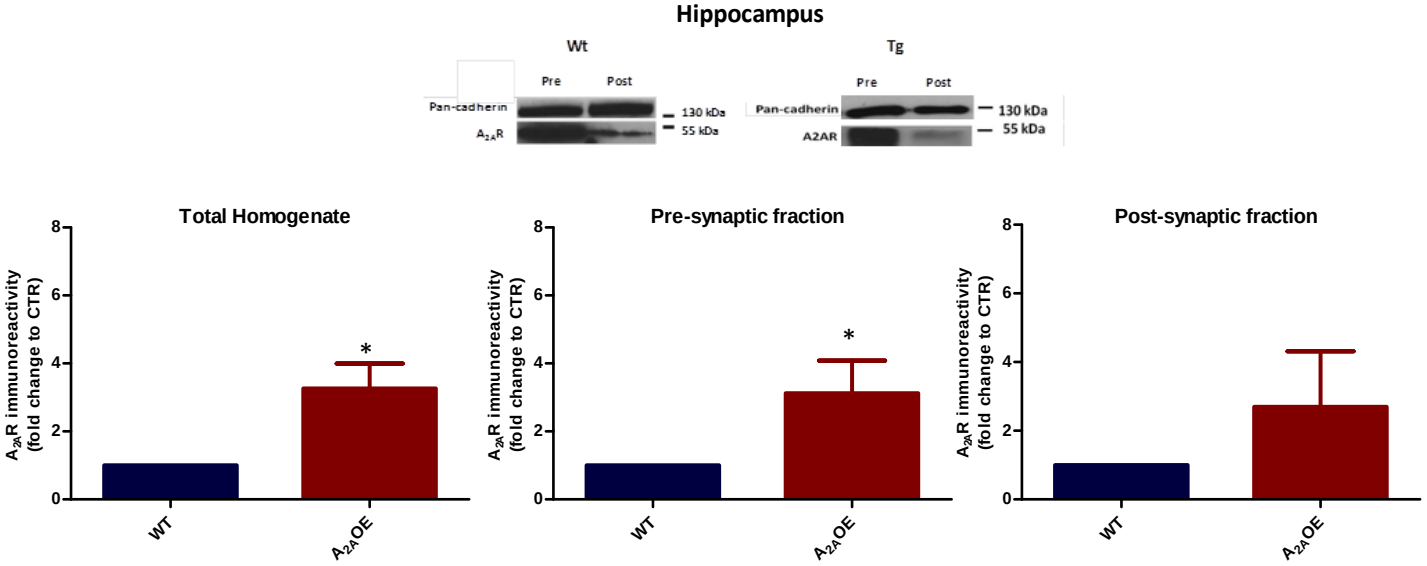


Figure 4.1 – (a) Western Blot of SNAP-25 and PSD-95 enrichment. (b) Preferential localization of the two markers in striatum (n=4) and hippocampus(n=5)

Studies revealed different actions of adenosine A_{2A} receptors and, these phenomena may be caused by a different subcellular localization of these receptors. In striatum these receptors are mainly post-synaptic [67] and, in contrast, extra-striatal adenosine A_{2A} receptors are mainly pre-synaptic [68].

The evaluation of subcellular localization of adenosine A_{2A} receptors in CamKII transgenic rats was performed. In total homogenates (input) of the hippocampus (Fig 4.2b) there is a significant increase of A_{2A} receptor levels in hippocampus of tg animals by 3.3±0.7 (n=4), but not in striatum. If we look into the subcellular fractions instead, we see that in hippocampus this increase in tg animals is a major contribution from the pre-synaptic fraction rather that the postsynaptic (3.1±0.9 fold increase in pre- *versus* 2.7±1.6 fold increase in postsynaptic fraction; n=5). However, in the striatum it is possible to observe that A_{2A} receptors are overexpressed mainly in the post-synaptic fraction (9.6±0.6 fold increase in pre- *versus* 2.5±0.6 fold increase in postsynaptic fraction) (n=2). Note that tg animals have a considerable interindividual variability of overexpression values.



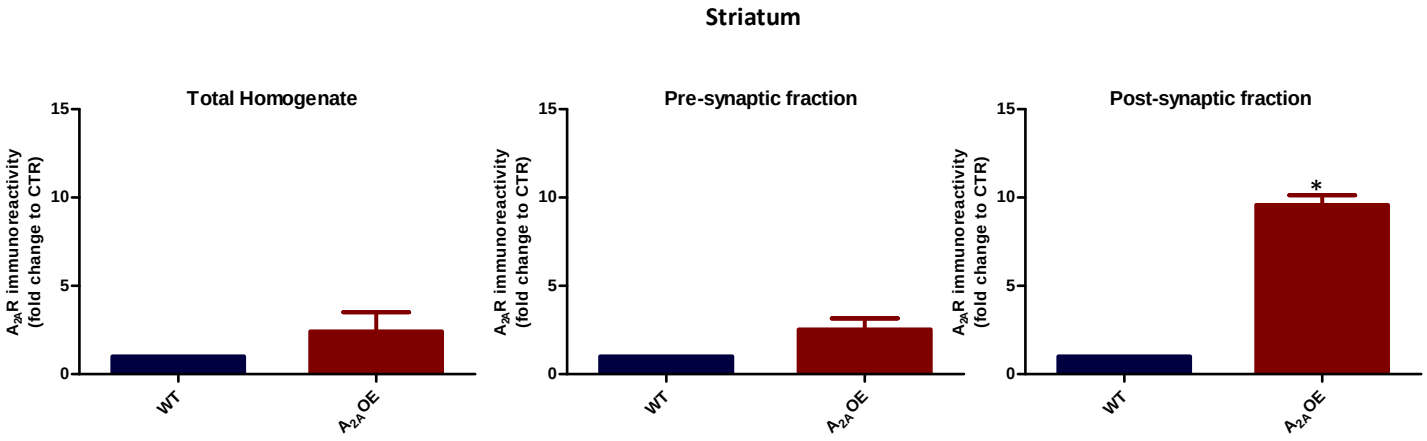


Figure 4.2 – (a) Representative Western Blot of A_{2A} receptor immunoreactivity in synaptic fractions of A_{2A} overexpressing tg animals and wt. (b) Adenosine A_{2A} receptor fold enrichment in different fractions in hippocampus and striatum. Specific immunoreactivity was normalized to the pan-cadherin levels within each fraction. Same loading volume was loaded for each lane (8µL for striatum, 18µL for hippocampus). This corresponds to: ± 1.5-3.5mg in tg, ±1.7-8.3mg in wt and ±4.4-5.3mg in tg and ±8.6-15mg in wt, in post synaptic fraction and pre-synaptic fraction, respectively in striatum. In hippocampus: ± 6-18mg in tg, ±3-10.1mg in wt and ±11-25mg in tg and ±8.3-22mg in wt, in post synaptic fraction and pre-synaptic fraction, respectively. Results are mean ±SEM of three experiments. *P<0.01 unpaired Student's T test

Moreover, it is important to show the ratio between the presence of A_{2A} receptors in the two fractions (Pre/post) in both areas and, in figure 4.3 it is possible to observe that in hippocampus the difference between the ratio of wt and transgenic (1.5±0.1 enrichment versus 2.1±0.4) is higher than in striatum (2.6±0.6% versus 2.8±1.5%).

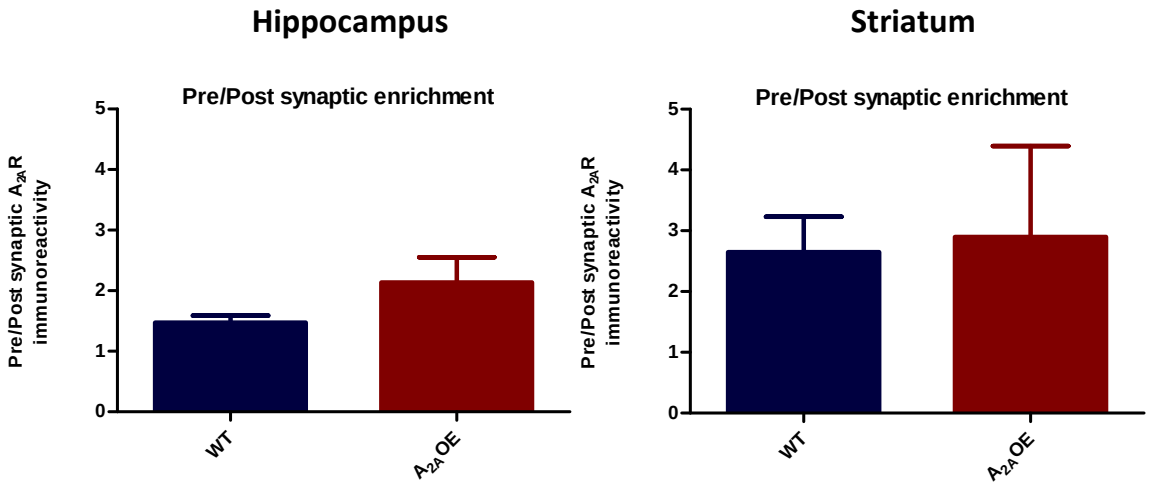


Figure 4.3 - Representative graphic of Pre/post synaptic fractions enrichment, in hippocampus (n=4) and striatum (n=3). Results are the mean ±SEM. Values were normalized to total input loaded.

Immunohistochemical profile of A_{2A} receptors in CamKII-hA_{2A}R rats

Adenosine A_{2A} receptors are not only confined to dopamine-rich areas of the rat brain, since thermocycling analysis shows that adenosine A_{2A} receptor mRNA is expressed also in the hippocampus (CA1, CA3 and dentate gyrus) and cerebral cortex [36]. But in these two areas they exhibit a very distinct pattern of expression, which is very low in physiological conditions and mainly concentrated in the nerve terminals [68]

Taking advantage of the transgenic rats overexpressing adenosine A_{2A} receptors in forebrain, that mimics ageing and pathological conditions, the A_{2A} distribution in terms of specific localization was analyzed.

For that, an immunohistochemistry analysis was performed, as described above (see Material and Methods), in whole brain 20µm slices. The slices were labeled with anti-MAP2 (microtubule-associated protein 2) that is a neuronal marker, anti-A_{2A} receptor and with Hoechst, for nuclear tagging.

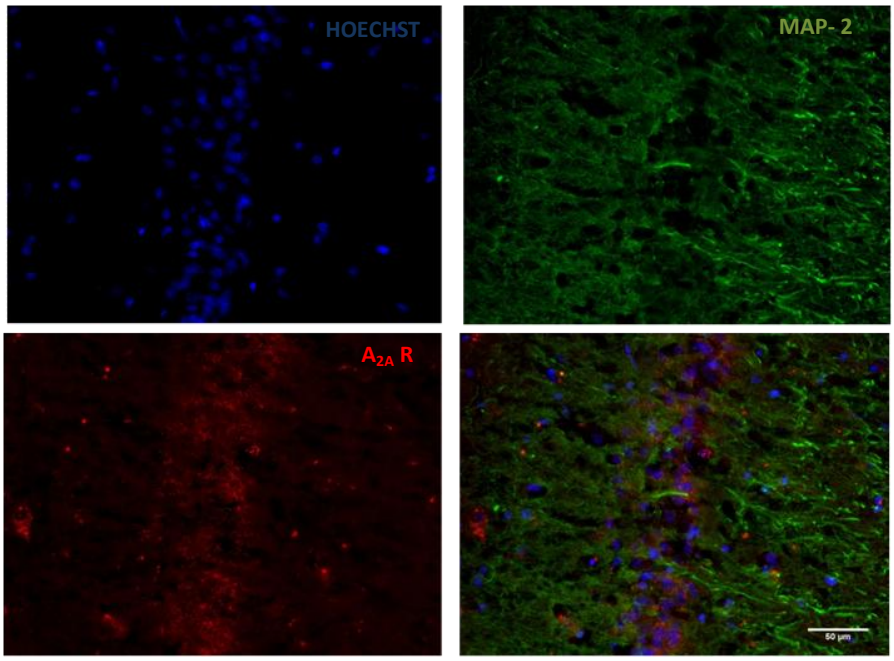


Figure 4.4 - Fluorescence immunohistochemistry images, illustrating CA1 area of the hippocampus in transgenic rats. This image was obtained by the author, with the microscope Zeiss Axiovert 200M, with the 40x amplification from a rat hippocampal slice section with 20µm. Neuronal nuclei are stained with Hoechst (blue), neurons are identified by green fluorescence using anti-MAP-2 and adenosine A_{2A} receptors by red fluorescence using anti-A_{2A}R.

Both antibodies used to reveal adenosine A_{2A} receptors (goat Anti-A_{2A}R from Santa Cruz or mouse Anti-A_{2A}R from Millipore) did not allow a consistent and viable conclusion, due to high background, poor signal and lack of specificity. We tested several different conditions, increasing fixation time to 1 hour and perfusion to 45 minutes, washing conditions (1 hour and a half for primary antibody and 1 hour for secondary); buffer compostion (Tween 0,1%+FBS 10%+1%BSA) was replaced by only FBS 10% and in other try by only BSA 3%) and even antibody dilution (1:100). Since this could be a consequence of low accessibility of this antibody to the epitopes, we added an additional permeabilization step with Triton X-100 0,05% for 15 minutes. After all these variations, we still could not obtain significant improvements in the images.

Immunocitochemistry in plated synaptosomes

A good alternative to overcome and enhance epitope exposure and study subcellular localization of adenosine A_{2A} receptors in transgenic rats is to use plated synaptosomes instead. Since adenosine A_{2A} receptors are localized predominantly at pre-synaptic nerve terminals in the hippocampus [68], to analyze the exactly localization of adenosine A_{2A} receptors is possible to use plated synaptosomes, as these structures represent nerve terminals. This is only possible provided that there are no changes in the levels of pre-synaptic nerve endings by overexpressing A_{2A} receptors. We checked this by confirming that synaptophysin levels in wt and tg does not change in total homogenates (see Figure 4.5).

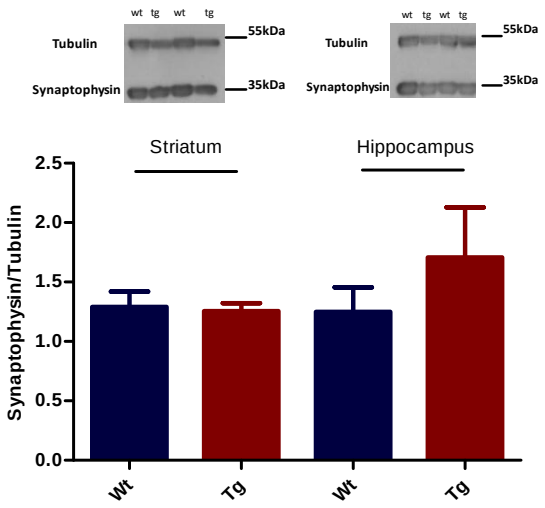
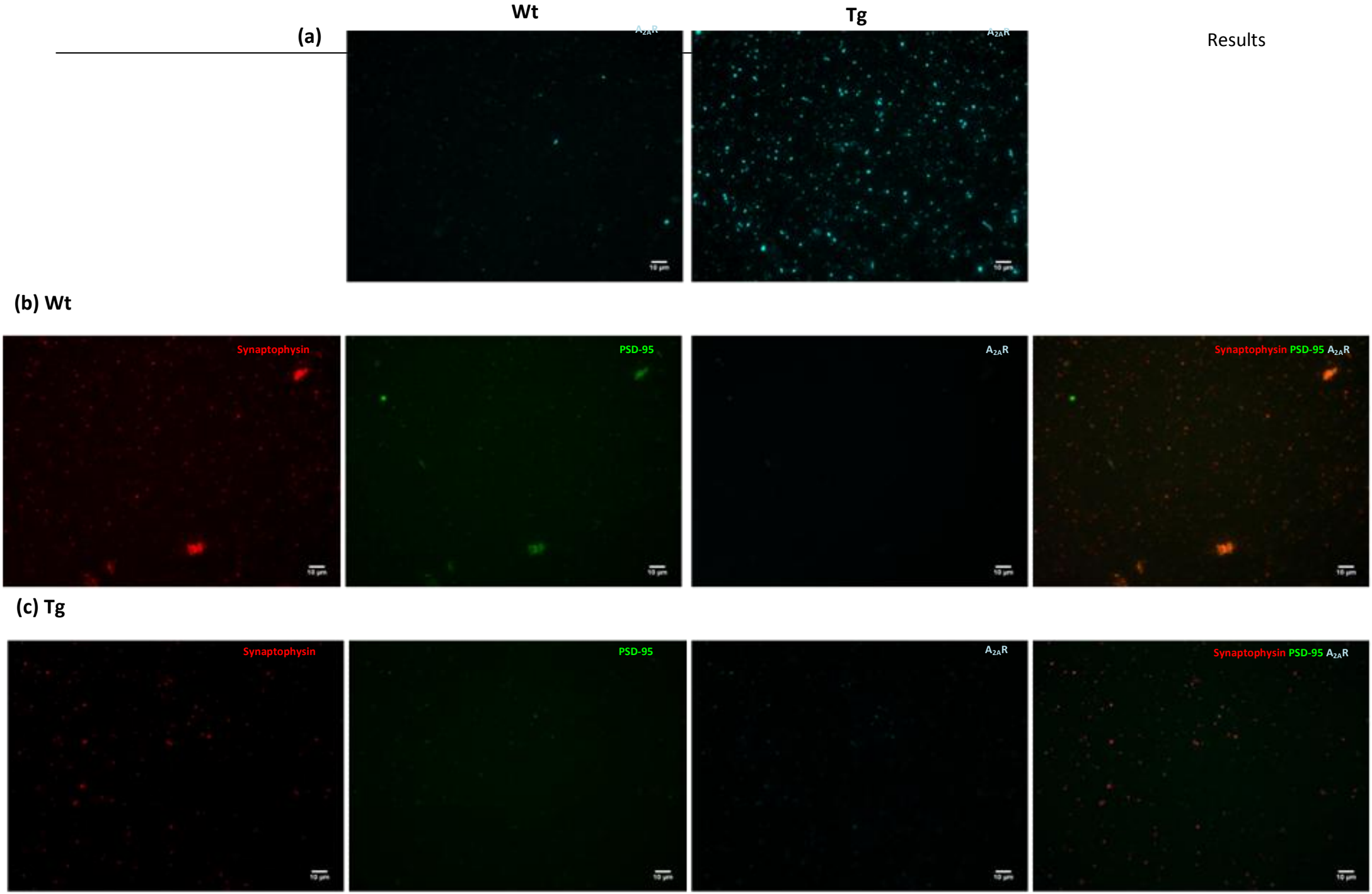


Figure 4.5 - Comparison of synaptophysin immunoreactivity both in striatum and hippocampus, between wt and tg rats. On the top is a representative Western Blot.

To perform subcellular localization analysis, hippocampal and striatal synaptosomes from transgenic and wild type rats (with 12-15 weeks) were isolated, as described in Material and Methods and stained with a mouse anti-synaptophysin (1:250) a pre-synaptic protein, goat anti-adenosine A_{2A} receptor (1:200) and rabbit anti-PSD-95 (1:250), a post-synaptic protein antibodies. The first one is a protein located in synaptic vesicles and consider as a pre-synaptic marker [69] so, the number of elements stained with synaptophysin was consider as the total number of nerve terminals.

Regarding adenosine A_{2A} receptors staining at hippocampus, the obtained images (figure 4.6) show a clear overexpression of A_{2A} receptors in plated synaptosomes: in transgenic, 53.8±7.8% (n=4) of the total number of nerve terminals are A_{2A} receptors positive whereas in wt, only 21.5±6.1% are A_{2A} receptor positive. From these, 10.5±4.0% (n=4) of the adenosine A_{2A} receptors are co-localized with synaptophysin in wt and 39.3±6.7% in transgenic (n=4). The percentage of A_{2A} receptors that co-localize with PSD95 is of 3.0±0.4% (n=4) in wt and of 10.8±2.3% (n=4).


Wt
Tg
(a)
A2AR
A2AR
(b) Wt
Synaptophysin
PSD-95
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PSD-95
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Synaptophysin PSD-95 A2AR

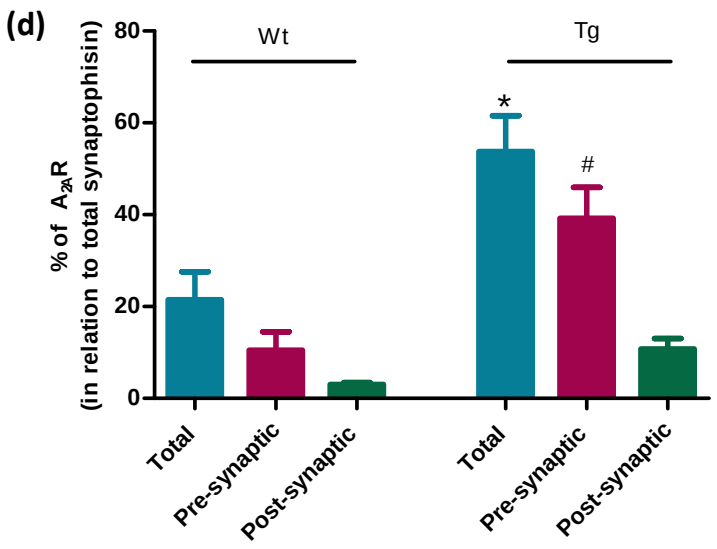
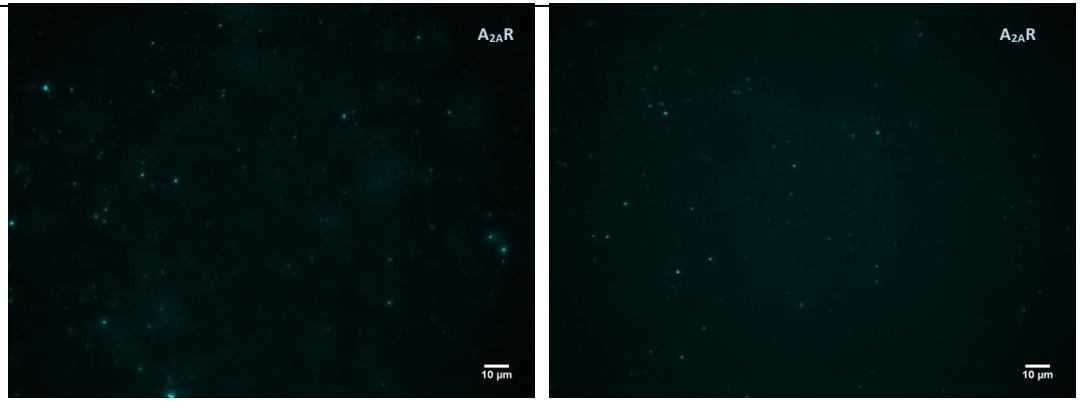


Figure 4.6- Co-localization of adenosine $A_{2A}R$ in hippocampus with synaptophysin and PSD-95. Fluorescence images were taken at Zeiss Axiovert 200M with 63x magnification. (a)Representative image of the total number of $A_{2A}R$ in Wt and transgenic; (b)Representative images of co-localization in wt rats, between synaptophysin (red) stained with mouse anti-synaptophysin antibody (1:250) and an Alexa Fluor 568 donkey anti-mouse (1:250), PSD-95 (green) stained with rabbit anti-PSD-95 (1:250) and an Alexa Fluor 488 donkey anti-rabbit and $A_{2A}R$ (cyan) stained with goat anti-$A_{2A}R$ (1:200) and Alexa Fluor 633 donkey anti-goat (1:250). (c) The same as (b) but in tg rats. The pink elements are pre-synaptic $A_{2A}R$. (d) Percentage of $A_{2A}R$ in each subcellular region, compared to total number of nerve terminals.Each bar is a mean±SEM of n=4 of n=4. *P<0.0001 and #P<0.05, compared to wt, calculating using one way ANOVA test plus a Bonferroni post hoc test.

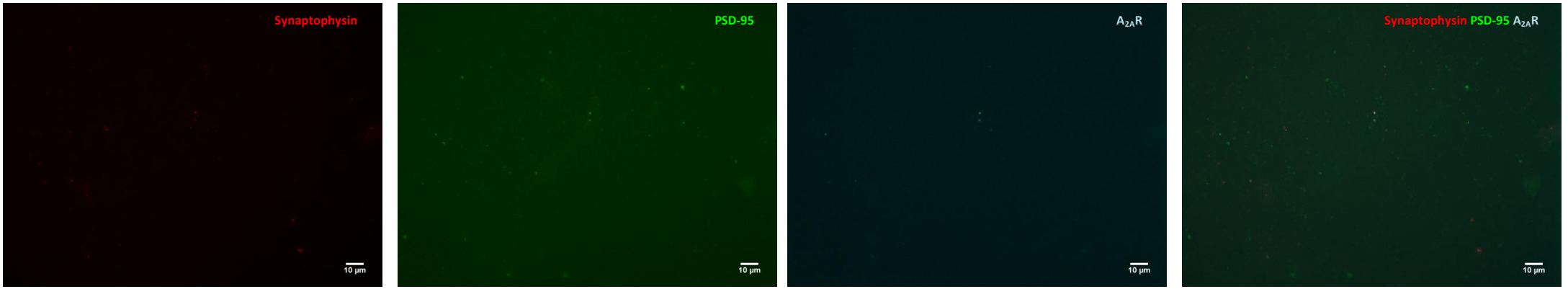
Striatum

The obtained results (figure 4.7) shows that the percentage of total adenosine A_{2A} receptors in wt is 19.5±8.5% (n=3) and in the A_{2A} transgenic rats 31.7±7.9% (n=3). The graphic shows a co-localization with synaptophysin of only 6.5±1.5% (n=3) in wild type and, in tg of 16.7±1.8% (n=3). The co-localization of A_{2A} with PSD-95 was of 6.0±1% (n=3) for wt and 7.3±4.3% (n=3) in tg

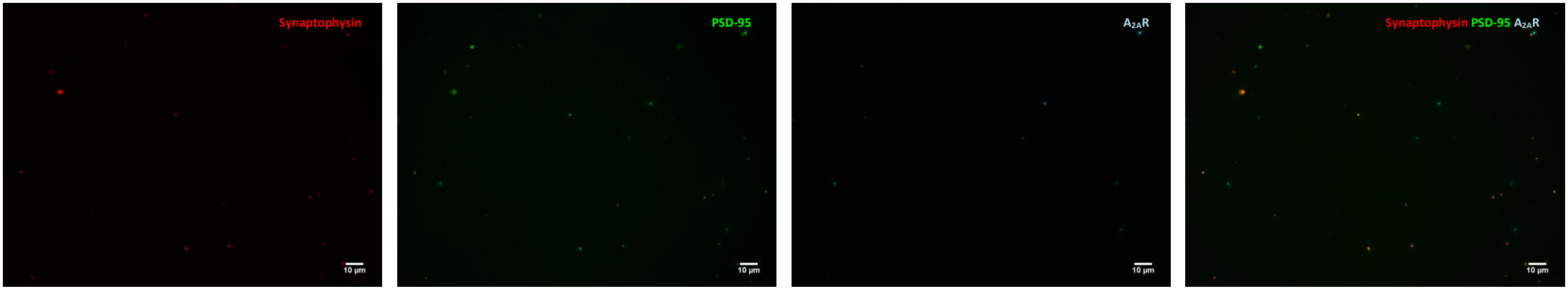
(a)


Wt
Tg
$A_{2A}R$
$A_{2A}R$
10 µm
10 µm

(b) Wt


Synaptophysin
PSD-95
$A_{2A}R$
Synaptophysin PSD-95 $A_{2A}R$
10 µm
10 µm
10 µm
10 µm

(c) Tg


Synaptophysin
PSD-95
$A_{2A}R$
Synaptophysin PSD-95 $A_{2A}R$
10 µm
10 µm
10 µm
10 µm

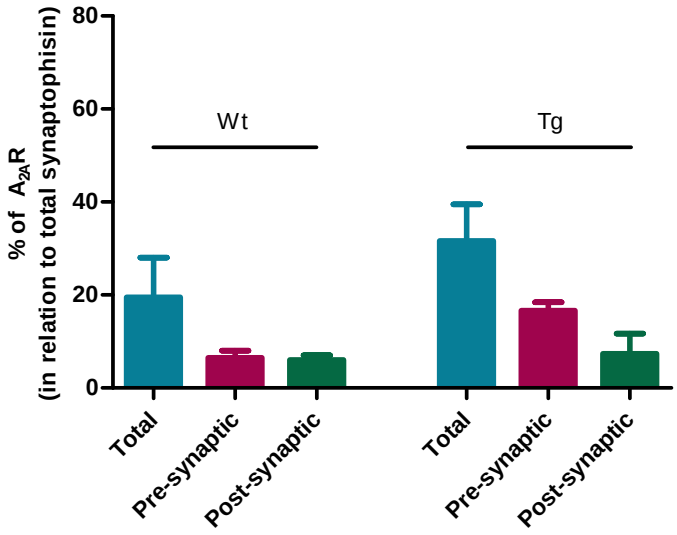


Figure 4.7- Co-localization of adenosine $A_{2A}R$ in striatum with synaptophysin and PSD-95. Fluorescence images were taken at Zeiss Axiovert 200M with 63x magnification. (a)Representative image of the total number of $A_{2A}R$ in wt and $A_{2A}OE$; (b)Representative images of co-localization in wt rats, between synaptophysin (red) stained with mouse anti-synaptophysin antibody (1:250) and an Alexa Fluor 568 donkey anti-mouse (1:250), PSD-95 (green) stained with rabbit anti-PSD-95 (1:250) and an Alexa Fluor 488 donkey anti-rabbit and $A_{2A}R$ (cyan) stained with goat anti-$A_{2A}R$ (1:200) and Alexa Fluor 633 donkey anti-goat (1:250). (c) The same as (b) but in tg rats. The pink elements are pre-synaptic $A_{2A}R$. (d) Percentage of $A_{2A}R$ in each subcellular region, compared to total number of nerve terminals. Each bar is the mean ±SEM of n=2-3

The percentage of postsynaptic density attached to synaptosomes was of 37.0±14.2% and 25.7±11.3%, in wt and tg, respectively for the hippocampus. Regarding striatum, this was of 51.5±8.5% and 38.5±17.6%, in wt and transgenic, respectively

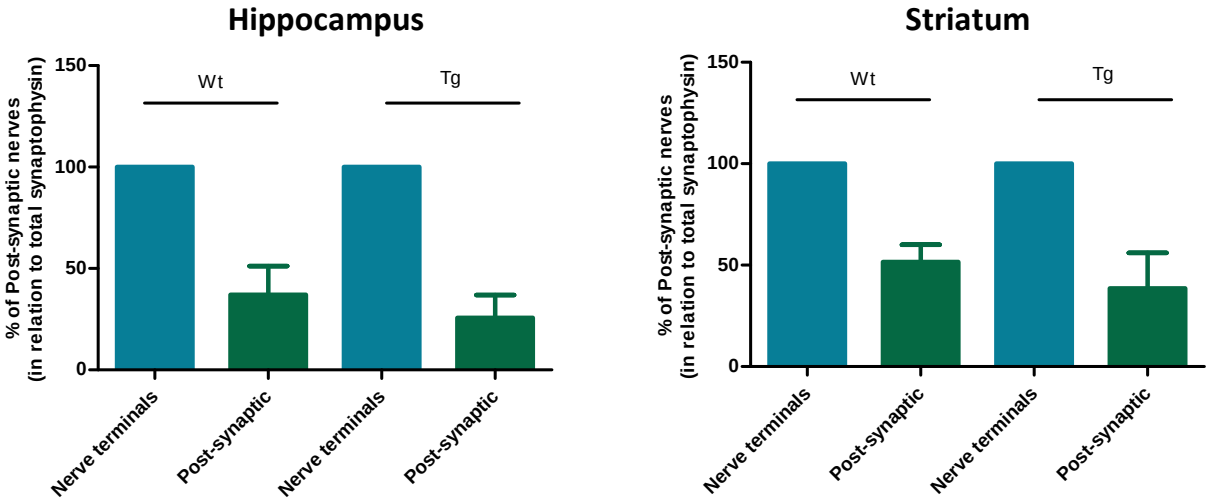


Figure 4.8- Percentage of elements stained with PSD-95. Blue bar represents the total number of nerve terminals (synaptophysin positive) and, green bar PSD-95 positive elements. The percentage of post-synaptic elements was obtained considering the total number of nerve terminals as 100% and the number of PSD-95 positive was normalized to that. (n=3-4 in hippocampus; n=2-4 in striatum)

Discussion

There is evidence of cortical and hippocampal upsurge of adenosine A_{2A} receptors expression/function associated to cognitive deficits [55], accompanied by behavioral deficits in hippocampal dependent tasks. It was not known whether these deficits are generated by adenosine A_{2A} receptors located pre- or post synaptically. So, taking advantage of transgenic rats overexpressing adenosine A_{2A} receptors in forebrain neurons, the main goal of this work was to determine the subcellular origin of overexpressed A_{2A} receptors. The main finding of this work is that adenosine A_{2A} receptors are overexpressed mainly at pre-synaptic level in hippocampus. In striatum, some overexpression was also observed but at a very much lower extent.

Ageing is associated with cognitive decline in both humans and animals. Among brain structures, the hippocampus appears to be particularly vulnerable to senescence and degeneration. Age and Alzheimer´s disease-related cognitive impairments are accompanied by structural and functional alterations in the hippocampus, that directly affect neural plasticity [70], leading to synaptic dysfunctions and, subsequently, memory deficits [71].

A_{2A} receptors are one of the main brain targets of the homeostatic neuromodulator adenosine [56]. A_{2A} are constitutively activated G-protein coupled-receptors, preferentially expressed by the striato-pallidal medium spiny striatal neurons [57]. They exhibit however a very distinct pattern of expression in the hippocampus and cortex where their expression is very low in physiological conditions [58]. Our team and others have found compelling evidence of cortical and hippocampal upsurge of A_{2A} receptor expression/function associated to cognitive deficits. Specifically, in the hippocampus of aged rats, A_{2A}R expression is nearly two fold than of young ones [58].

In the hippocampus, the A_{2A} receptors-dependent activation of glutamate release becomes more pronounced as ageing progresses and shifts from a protein kinase C-mediated signaling to a cAMP-dependent effect [58]. This is accompanied by clear behavioral deficits in hippocampal-dependent tasks, such as spatial memory in rats. Accordingly, rats overexpressing hippocampal A_{2A} receptors also exhibit behavioral deficits including spatial memory defects as well as LTP impairments [59]. Interestingly, other

detrimental conditions associated to cognitive impairments, such as hypoxia, diabetes or epilepsy share similar $A_{2A}R$ overactivation [72] see [15] for review.

In physiological conditions A_{2A} receptors are at low density in hippocampus and are expressed both pre- and post- synaptically [68] but mainly at pre-synaptic level, modulating the evoked release of neurotransmitters, controlling the release of neurotransmitters, either glutamate [37], GABA [73], acetylcholine or serotonin and, at post-synaptic level control responsiveness in hippocampus [74]

Nevertheless, the exact subcellular origin of the A_{2A} receptors contributing to disease was never assessed. We now took advantage of a novel model of transgenic rats overexpressing adenosine A_{2A} receptors in forebrain neurons, under CAMKII promoter control. The goal was to find whether these deficits are related with a pre- or a post-synaptic overexpression that mimics pathophysiological conditions.

A_{2A} receptors in transgenic animals are predominantly present in the pre-synaptic fraction of hippocampal neurons.

There are previous evidences that different actions of adenosine A_{2A} receptors may be caused by different subcellular localization of these receptors [67]. By performing a fractionation protocol, it was observed by Western Blotting an A_{2A} receptor preferential co-localization with a pre-synaptic protein, SNAP-25, than with a post-synaptic protein, PSD-95 in the hippocampus.

Previous studies in striatum, showed that adenosine A_{2A} receptors were identified mainly at post-synaptic level [49] in GABAergic neurons, in the same cells that express D_2 dopamine receptors [75]. There is also an evidence for the existence of post-synaptic mechanisms in the control of glutamatergic neurotransmission.

With this work is possible to conclude that A_{2A} receptors in striatum are mainly present in post-synaptic fraction, as expected. However, we also observed a band in the pre-synaptic fraction. This is probably due to the adenosine A_{2A} receptors present in glutamatergic terminals from cortex, where they heteromerize with A_1 receptors and have a stimulatory role, facilitating glutamate release. [75]. One note to mention is that, in

striatum the experiment would be more specific if PSD-95 were replaced by gephyrin since, this last is a marker characteristic of GABaergic synapses [76].

Then we wanted to confirm whether A_{2A} receptors co-localize with pre-synaptic proteins by using imaging. We first tried to assess the profile of overexpression in brain sections. Usually, İmmunohistochemical for A_{2A} receptors is technically difficult in native tissue of cortical areas, due to its low expression and poor epitope access. It was thought that using an overexpression model would allow to bypass these difficulties. Therefore we tried to present innovative results about the evaluation of the profile of adenosine A_{2A} overexpression in a whole brain slice. Nevertheless and is spite of our efforts to change several variables such as nature of antibody, time, blocking and permeabilization steps, A_{2A} receptors in whole slice proved difficult to tag. Although in denaturing conditions or in cell cultures the antibodies are very efficient, in native tissue this is not the case.

Using plated synaptosomes is possible to confirm co-localization with synaptophysin.

In neurochemical research, it has been accepted to divide neuronal parts of synapse into two: pre- and post-synaptic [63]. Since the early 1960s, synaptosomes have an extensively use as a model of pre-synaptic neuron. These structures contain the complete pre-synaptic terminal, along the post-synaptic membrane and the post-synaptic density (PSD) [64]. There are a lot of studies performed in synaptosomes, namely metabolism and bioenergetics and neurotransmitter release [77].

These structures are a good approach taking into account that adenosine A_{2A} receptors, in physiological conditions are mainly present in nerve terminals [68]. Their co-localization in transgenic animals was tested. Synaptosomes were stained with pre- and post-synaptic markers, synaptophysin and PSD-95, respectively, and for A_{2A} receptors, in striatum and hippocampus. The numbers of elements, stained with synaptophysin were considered as the total number of nerve terminals and, as this protein did not vary between two genotypes, values in wild type and transgenic can be compared in relation to synaptophysin.

With this experiment it was possible to conclude that, in hippocampus, adenosine A_{2A} receptors are mainly co-localized with synaptophysin, both in wild type and transgenic rats, being the percentage of co-localization higher in transgenic rats, due to $A_{2A}R$ overexpression. In striatum, the percentage of co-localization of A_{2A} receptors with synaptophysin was residual and probably refers to cortical terminals projecting onto striatum. The percentage of PSD 95 positive signal is probably from postsynaptic density attached to pre-synaptic fraction, as shown before [78].

In conclusion, the results suggest a preferential pre-synaptic localization of adenosine A_{2A} receptors, in hippocampus of transgenic rats which display LTP impairments and deficits in hippocampal dependent tasks. These results are also in agreement with what is observed upon ageing or stress [55] where this pre-synaptic increase was pointed to be the trigger for a shift in adenosine modulation. This CAMKII driven overexpression seems to mimic a pathophysiological condition regarding functional effects and pre-synaptic localization of A_{2A} receptors.

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